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Metal Regulation of Siderophore Synthesis in *Pseudomonas aeruginosa* and Functional Effects of Siderophore-Metal Complexes

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***Pseudomonas aeruginosa* synthesizes two siderophores, pyochelin and pyoverdin, characterized by widely different structures, physicochemical properties, and affinities for Fe(III). Titration experiments showed that pyochelin, which is endowed with a relatively low affinity for Fe(III), binds other transition metals, such as Cu(II), Co(II), Mo(VI), and Ni(II), with appreciable affinity. In line with these observations, Fe(III) and Co(II) at 10 μM or Mo(VI), Ni(II), and Cu(II) at 100 μM repressed pyochelin synthesis and reduced expression of iron-regulated outer membrane proteins of 75, 68, and 14 kDa. In contrast, pyoverdin synthesis and expression of the 80-kDa receptor protein were affected only by Fe(III). All of the metals tested, except Mo(VI), significantly promoted *P. aeruginosa* growth in metal-poor medium; Mo(VI), Ni(II), and Co(II) were more efficient as pyochelin complexes than the free metal ions and the siderophore. The observed correlation between the affinity of pyochelin for Fe(III), Co(II), and Mo(VI) and the functional effects of these metals indicates that pyochelin may play a role in their delivery to *P. aeruginosa*.**

A number of transition metals are needed by bacteria as vital constituents, but their availability in the environment may not suffice to support microbial growth (16, 19, 38). In fact, although these metals can exist as the free, uncomplexed ion or in a complexed form, the free metal ion may not be the dominant species even in the absence of ligands. This is due to indirect complex formation (14) resulting from the generation of poorly soluble oxides or oxy-hydroxy polymers under aerobic conditions at neutral pH, i.e., under physiological conditions (6, 14). Iron is a type case in this respect; it has a major metabolic role in almost all forms of life but is poorly soluble since both ferric and ferrous salts rapidly hydrolyze to insoluble polymeric hydroxides at pH values approaching neutrality. Thus, the maximum concentration of Fe(III) free in solution at a pH close to 7 has been estimated to be about 10^{-18} M (35), a value too low for the microbial requirements.

To counter the effect of iron limitation in the environment or within the mammalian host tissues (8, 37), most microorganisms have developed high-affinity systems for iron uptake, consisting of low-molecular-weight chelators, called siderophores, and their cognate membrane receptors (19, 30). Such iron acquisition systems have been hypothesized to be involved also in the transport of other essential transition metals required for the activity of metalloenzymes, like molybdenum, cobalt, nickel, and copper (19, 27, 31, 38), but the experimental evidence has so far not been conclusive.

Pseudomonas aeruginosa is an opportunistic pathogen which is faced with a stringent iron requirement for multiplication both in the infected host and in the environment (8). Like other fluorescent pseudomonads, it has a strict respiratory type of metabolism, usually with oxygen as the terminal electron acceptor, and is unable to grow at acid pH (32). These metabolic requirements justify the need for multiple, efficient iron uptake systems. The strain *P. aeruginosa* ATCC 15692 (PAO1) has been shown to synthesize two distinct siderophores, pyochelin and pyoverdin.

Pyochelin (10) is an unusual siderophore in that it is a poorly water-soluble, low-molecular-weight (325-Da) thiazoline derivative [2-(2-*o*-hydroxy-phenyl-2-thiazolin-4-yl)-3-methylthiazolidine-4-carboxylic acid] (11) which binds Fe(III) with a stoichiometry of two molecules per iron atom and a remarkably low stability constant of approximately 2×10^5 M^{-2} (10). Ferripyochelin transport has been shown to involve two distinct outer membrane proteins (OMPs) of 14 (34) and 75 kDa (18), the latter characterized by a higher affinity, i.e., operating at Fe(III) concentrations 5- to 10-fold lower than the former (18).

Pyoverdin (4, 9) is a more complex molecule (approximately 1,500 Da) consisting of an 8-hydroxyquinoline derivative, which forms a fluorescent chromophore, and an octapeptide, which contains two residues of N^5-hydroxyornithine (39). The hydroxamate groups formed by these two residues participate in the binding of iron together with the catecholate group of the chromophore. Pyoverdin is a very water-soluble molecule and a powerful chelator of ferric iron, which is bound with a stoichiometry of 1:1 (9, 39) and a stability constant of approximately 10^{24} M^{-1} at neutral pH (26, 39). Both features favor Fe(III) binding to pyoverdin with respect to pyochelin (10, 27). Two high-molecular-mass OMPs of 80 (28) and 90 kDa (33) have been shown to function in ferripyoverdin uptake.

The strikingly different affinities and stoichiometries of the two *P. aeruginosa* siderophores for Fe(III) led us to assume that these molecules may also differ in their binding properties of other transition metals. Indeed, pyochelin is known to form complexes with zinc ions (13). Hence, while the fundamental role of pyoverdin and other fluorescent sidero-

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phores in iron uptake by *Pseudomonas* spp. is undisputed, the actual biological significance of pyochelin as a specific Fe(III) carrier may be questioned.

To investigate whether the two *P. aeruginosa* siderophores possess a sequestering activity towards essential transition metals other than iron, we determined the effect of metal addition on *P. aeruginosa* PAO1 growth in a metal-poor medium, on pyochelin and pyoverdin biosynthesis, and on the expression of OMPs involved in siderophore-mediated iron uptake. The data obtained show that growth, pyochelin production, and expression of these specific OMPs are modulated by transition metals other than Fe(III) and point to a significant affinity of pyochelin for such cations, an indication which was substantiated by direct measurements of metal binding. Hence, a possible involvement of pyochelin in the transport of different transition metals has been proposed.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. aeruginosa* PAO1 (ATCC 15692) was obtained from the American Type Culture Collection and stored as frozen culture at −80°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) and 10% glycerol. Cultures were routinely grown in DCAA, containing 0.5% Casamino Acids (Difco) in deionized, double-distilled water, made metal free by treatment with Chelex 100 resin (Bio-Rad Laboratories, Richmond, Calif.). According to the specifications of the manufacturer, this matrix is characterized by the following order of selectivity for divalent and trivalent cations: Cu(II) > Fe(III) > Cr(III) > Ni(II) > Zn(II) > Co(II) > Cd(II) > Fe(II) > Mn(II). The activated resin was added to the Casamino Acids solution (5 g of resin per liter of medium) and the pH was adjusted to 7.4 by the addition of a few drops of 1 N NaOH. After 20 h of moderate stirring at 4°C, the resin was removed by filtration through Whatman (Clifton, N.J.) no. 1 filter paper and the medium was sterilized by autoclaving. The medium was supplemented aseptically with 0.4 mM $MgCl_2$ immediately before inoculation. After Chelex 100 treatment, the metal content of DCAA (with respect to iron, copper, nickel, cobalt, zinc, molybdenum, chromium, manganese, and cadmium), was determined to be <0.1 μM by means of an atomic absorption spectrometer (Perkin-Elmer model 360). Alternatively, the M9 minimal medium (17) was used after supplementation with 20 mM sodium succinate as the carbon source. The M9 basal solution was also treated with Chelex 100 as described above. When required, media were made solid by the addition of 10 g of Agar N.1 per liter (Oxoid Ltd., Basingstoke, England).

To deplete cells of the intracellular metal pools, bacteria were first cultured on M9-succinate agar medium, suspended in a small volume of sterile, double-distilled water to yield an A_{620} of around 0.5, and inoculated in DCAA (approximately 10^6 CFU/ml). The culture was incubated at 37°C in a rotary shaker at 250 rpm until the late exponential phase was reached. This culture was subsequently used as the inoculum to achieve a final concentration of about 10^6 CFU/ml.

Chemicals and glassware. Metal salts were obtained from Merck (Darmstadt, Germany) in the following forms: $FeCl_3 \cdot 6H_2O$ (iron content, >99.9%); $MnSO_4 \cdot H_2O$ (iron content, <0.002%); $NiSO_4 \cdot 6H_2O$ (iron content, <0.001%); $CoSO_4 \cdot 7H_2O$ (iron content, <0.0005%); $ZnSO_4 \cdot 7H_2O$ (iron content, <0.001%); $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (iron content, <0.002%). $CuSO_4 \cdot 5H_2O$ (iron content, <0.003%) was purchased from Farmitalia Carlo Erba (Milano, Italy).

All glassware was rendered iron free by 24 h of treatment with 0.1 N HCl, rinsed once in distilled water, and finally rinsed in deionized, double-distilled water before sterilization. All media and solutions were prepared with deionized, double-distilled water.

Extraction and characterization of siderophores. Minor modifications of the methods proposed by Cox and Graham (10) and Meyer and Abdallah (26) were adopted for the extraction and detection of pyochelin and pyoverdin, respectively. Pyochelin was determined by ethyl acetate extraction of acidified supernatants obtained after centrifugation (5,000 × *g*, 30 min, 4°C) of 20-h bacterial cultures in DCAA. Briefly, the spent medium was adjusted to pH 1.5 to 2.0 with 1.0 N HCl and extracted twice with 0.4 volume of ethyl acetate. After evaporation of the organic phase under vacuum, the dry residue was resuspended in a small volume of methanol and applied in triplicate to a Silica Gel G thin-layer chromatography (TLC) plate in chloroform-acetic acid-ethanol (90:5:2.5) as the development solvent. Pyochelin from TLC plates was qualitatively characterized by (i) yellow-green fluorescence emission under UV light (10), (ii) iron-binding capacity resulting in red-brown spots when sprayed with 0.1 M $FeCl_3$ in 0.1 M HCl (10), (iii) specific reactivity of *N*-methylthiazolidine rings yielding black spots when sprayed with ammoniacal silver nitrate reagent (2, 20), and (iv) chromatographic mobility (R_f = 0.35 to 0.40 in the above development solvent [3]). Quantitative determinations of pyochelin were performed by scraping the fluorescent spots from TLC plates loaded with 50-ml culture extracts. Pyochelin was eluted from the silica gel by extracting twice with methanol. The methanol extracts were dried under vacuum, and the amount of pyochelin was determined by spectrophotometric titrations of the deferri- and iron-saturated form in the 200- to 600-nm wavelength range (10).

Pyoverdin production was qualitatively detected by fluorescence spectra in the 350- to 500-nm wavelength range of culture supernatants diluted 50-fold in 100 mM Tris-HCl (pH 7.0) by using a Perkin-Elmer LS50 fluorimeter; pyoverdin displays characteristic excitation and emission maxima at 405 and 455 nm, respectively (9, 26). The pyoverdin concentration in culture supernatants was estimated by spectrophotometric titrations of the Fe(III) complex at 475 nm with ferric citrate (26). The culture supernatants were supplemented with 0.1 volume of 100 mM Tris-HCl buffer (pH 7.0), and the pyoverdin concentration was determined by measurements of A_{475} after addition of increasing amounts of ferric citrate, up to the saturation end point; the reference, consisting of sterile DCAA, was treated as described above. The Csàky test (12) for hydroxamates was also carried out to quantify the concentration of hydroxylamino nitrogen in liquid cultures, by using a standard curve obtained with known quantities of hydroxylamino hydrochloride. In our experiments, 1 mol of pyoverdin corresponded to approximately 2 mol of hydroxamates, because of the presence of two N^5-hydroxyornithine residues per pyoverdin molecule (39).

Large-scale preparation of pyochelin and pyoverdin. Large-scale preparations of pyochelin were performed in stirred 1-liter flasks, filled with 250 ml of DCAA, and the siderophore was extracted as described by Cox and Graham (10). Pyochelin dissolved in methanol was applied to Whatman no. 1 filter paper, and chromatograms were developed ascending in water-acetic acid (90:1). After development and drying, the paper chromatogram was viewed under UV light, and the fluorescent bands were marked, cut out, and eluted in methanol. The methanol solution was concentrated under

vacuum and then chromatographed on a thin layer of Silica Gel G with chloroform-acetic acid-ethanol (90:5:2.5) as the development solvent. The chromatogram was observed under UV light, and the fluorescent band containing pyochelin was scraped from the plate and eluted with methanol for quantitative determination.

Large-scale purification and preparation of iron-free pyoverdin was performed as previously described (9, 26) by using M9 minimal medium supplemented with sodium succinate (20 mM) as the carbon source.

Preparation of metal complexes of pyochelin and pyoverdin. A methanol solution of pyochelin was evaporated under vacuum, and the dry residue was resuspended in ethanol to yield a final siderophore concentration of 40 mM. Pyoverdin was eluted from a Sephadex G25 column with deionized water, purified by high-performance liquid chromatography (9), concentrated by freeze-drying, and resuspended at 20 mM in deionized, double-distilled water. Equal volumes of metal salts (20 mM in water) and siderophores (40 mM pyochelin and 20 mM pyoverdin) were mixed to yield 10 mM pyochelin- and pyoverdin-metal complexes in 2:1 and 1:1 ratios, respectively. Mixtures were left standing at 4°C for 12 h, sterilized by filtration through Millipore membranes (pore size, 0.45 μm), and diluted 1,000-fold in DCAA. The flasks containing pyochelin and its metal complexes were prewarmed for 1 h at 42°C under vigorous agitation to facilitate ethanol evaporation, prior to the addition of the bacterial inocula.

Measurement of bacterial growth rates. *P. aeruginosa* PAO1 from succinate M9 minimal medium agar plates was inoculated into 25 ml of DCAA and grown overnight at 37°C with vigorous shaking to deplete cells of essential metals. Bacterial cells were centrifuged (20 min, 2,500 × g, 37°C; in a Heraeus Omnifuge 2.0 RS centrifuge) and resuspended in prewarmed DCAA to an A_{620} of 1.0, and 0.1-ml aliquots of bacterial suspension were used to inoculate 25 ml of prewarmed DCAA in 350-ml Erlenmeyer flasks. Cultures were incubated at 37°C with aeration in an orbital shaker (New Brunswick model G25) at 250 rpm, and 1-ml aliquots were removed at hourly intervals for measurements of optical density at 620 nm. The rate of biomass increase was estimated for 6 h after the inoculum addition, and the generation time was determined as previously described (40). The *t* test was applied to assess the significance of the differences between the mean generation times.

Analysis of OMPs. Exponentially growing bacteria (approximately 10^{10} cells) were harvested by centrifugation (20 min, 2,500 × *g*, 4°C), washed twice with ice-cold phosphate-buffered saline (10 mM phosphate buffer, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ [pH 7.2]), and resuspended in 1 ml of 30 mM Tris-HCl buffer (pH 8.0). Cell envelopes were disrupted by ultrasonication (three 60-s pulses) in ice, and bacterial debris were removed by centrifugation (20 min, 2,000 × *g*, 4°C). Sarkosyl (*N*-lauroyl-sarcosine, sodium salt; Sigma Chemical Co., St. Louis, Mo.) (20% [wt/vol]) and the protease inhibitor phenylmethylsulfonyl fluoride were added to the supernatants to a final concentration of 2% (wt/vol) and 1 mM, respectively. The mixtures were incubated at room temperature for 30 min and then centrifuged at 38,000 × *g* for 1 h at 4°C. The membrane pellets were washed with 30 mM Tris-HCl (pH 8.0), resuspended in a small volume of the same buffer, and stored at −20°C. Membrane preparations were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22) with 10% separating gels and 5% stacking gels. The electrophoresis buffer was 25 mM Tris-HCl (pH 8.3) containing 192 mM glycine and 1% (wt/vol) SDS. After electrophoresis, gels were stained with Coomassie brilliant blue, destained, and photographed.

Spectrophotometric titration of pyochelin by metal addition. Absorption spectra in the visible and UV ranges were measured at 20°C with a Cary 2200 spectrophotometer. Metal stock solutions were prepared by dissolving their salts in 3 mM HCl (pH 2.5). Titrations were performed by the stepwise addition of the metal stock solution into the sample (containing 1 ml of pyochelin in methanol at pH 2.5) and into the reference cuvette (containing 1 ml of methanol at pH 2.5). Under the solvent condition chosen, pyochelin solubility is high and the absorption spectrum of its complex with Fe(III) has been reported (10, 11). The acidic pH also favors the solubility of the metal ions, in particular Fe(III).

RESULTS

Effect of different transition metals on siderophore biosynthesis by *P. aeruginosa* PAO1. The effect of metal ions on growth and expression of iron uptake systems by *P. aeruginosa* PAO1 was studied by using a Casamino Acids medium treated with Chelex-100 (DCAA). This treatment is known to result in an overall metal ion deficiency (see Materials and Methods).

Initially, the production of pyochelin and pyoverdin by *P. aeruginosa* PAO1 was monitored during a 30-h incubation period at 37°C in 50-ml quantities of DCAA. In accordance with previous results (7, 10, 26), the coordinate production of both siderophores paralleled growth and remained constant until the end of the logarithmic phase, at which time pyochelin and pyoverdin reached concentrations of 6 and 80 μM, respectively (data not shown).

Thereafter, the effect of transition metals on pyoverdin and pyochelin production was investigated by testing the dose-response effect of Fe(III), Cu(II), Ni(II), Co(II), Zn(II), and Mo(VI) on growth yields and siderophore biosynthesis (Fig. 1). Fe(III) added at 5 μM caused a complete repression of pyoverdin and pyochelin synthesis, in agreement with previous observations (7, 10, 26). Pyoverdin production was not affected by the other metals tested at concentrations up to 10 μM and was repressed only slightly at higher concentrations (50 to 100 μM, depending upon the metal). In contrast, pyochelin synthesis was significantly reduced. The most striking effect was obtained with Co(II), which behaved similarly to Fe(III) in that it totally repressed pyochelin production at concentrations of 5 to 10 μM. The least effective metals were Zn(II), which yielded approximately 25% repression of pyochelin biosynthesis at a concentration of 100 μM, and Mn(II) (data not shown), which had practically no effect at the same concentration.

Growth-promoting activity of metals and their pyoverdin and pyochelin complexes. To determine the effect of metals and their siderophore complexes on *P. aeruginosa* PAO1 growth, experiments were carried out in which the metal-poor medium (DCAA) was supplemented with metal salts or the metal siderophore complexes at a final concentration of 10 μM. This concentration was chosen because it was higher than the commonly accepted threshold of passive diffusion of the free metal. As a control, the effect of the siderophores was also tested (Table 1). All metals except Mo(VI) promoted growth significantly, albeit to different extents. The highest growth-stimulating activity was shown by Fe(III), as indicated by the decrease in the mean generation time from 74.5 to 51.7 min ($t = 9.815$; $P = 0.001$). It should be stressed that Fe(III) at 10 μM completely represses the synthesis of both siderophores. The other metals, namely, Cu(II), Ni(II),

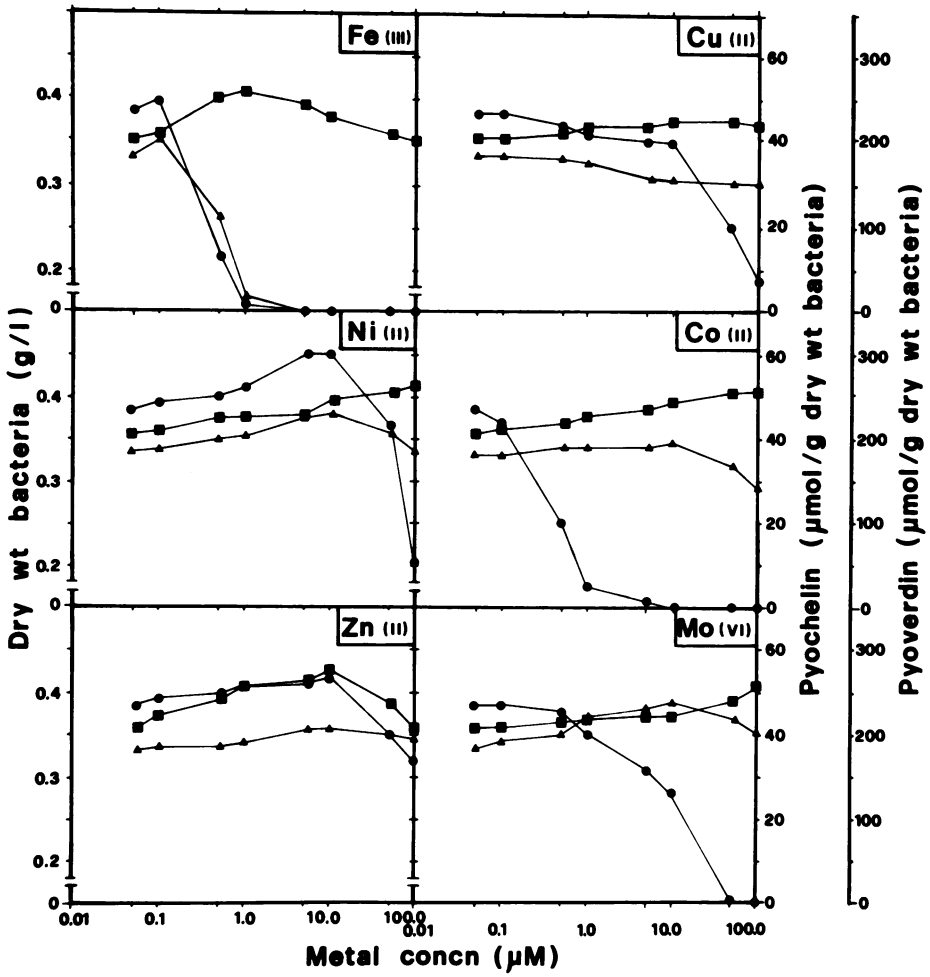

FIG. 1. Dose-response effect of various transition metals on growth yield and siderophore production by *P. aeruginosa* PAO1 in metal-poor medium. Bacteria were grown for 20 h at 37°C in 50 ml of DCAA. The abscissa shows the initial concentration of metals added to the DCAA. Symbols: ■, dry weight of bacteria (in grams per liter); ▲, pyoverdin yields (in micromoles per gram [dry weight] of bacteria); ●, pyochelin yields (in micromoles per gram [dry weight] of bacteria).

Co(II), and Zn(II), caused significant reductions of the mean generation time, ranging from 58.7 min for Co(II) ($t = 6.639$; $P = 0.002$) to 69.7 min for Cu(II) ($t = 2.633$; $P = 0.058$). The addition of either siderophore, at a high concentration with respect to that of the inoculum, also significantly reduced the mean generation time of *P. aeruginosa* in DCAA ($t = 3.802$, $P = 0.019$ [for pyoverdin]; $t = 6.379$, $P = 0.003$ [for pyochelin]), presumably because of metal ion scavenging from the medium. The complexes of pyoverdin with Fe(III), Cu(II), Ni(II), and Mo(VI) caused significant growth stimulation when compared with that of the respective metal salts ($2.760 < t < 11.154$; $0.001 < P < 0.050$). In the case of pyochelin, such a growth stimulation was displayed by the complexes with Cu(II), Ni(II), and Mo(VI) ($5.155 < t < 10.315$; $0.000 < P < 0.006$) and, to a lesser extent, by those with Co(II), Zn(II), and Fe(III) ($1.394 < t < 2.706$; $0.053 < P < 0.236$). A very evident effect was obtained with the pyochelin complex with Mo(VI), which reduced the mean generation time to approximately 50 min ($t = 8.502$; $P = 0.001$). However, if one compares the growth-promoting effect of the siderophore-metal complexes with that of the isolated components, that of the siderophores alone, and that of the respective metal salts, only the pyoverdin com-

TABLE 1. Effect of various metals and their complexes with pyoverdin and pyochelin on *P. aeruginosa* PAO1 growth in metal-poor medium (DCAA)

Metal	Mean generation time (min ± SD)[a] in DCAA supplemented with:		
	Metal[b]	Metal-pyoverdin[c]	Metal-pyochelin[d]
None	74.5 ± 2.6	64.5 ± 3.7	63.8 ± 1.2
Fe(III)	51.7 ± 3.0	45.1 ± 2.8	48.0 ± 3.4
Cu(II)	69.7 ± 1.7	62.2 ± 2.3	63.4 ± 1.2
Ni(II)	65.3 ± 0.7	60.3 ± 1.4	57.2 ± 1.1
Co(II)	58.7 ± 3.1	58.0 ± 1.4	53.2 ± 2.4
Zn(II)	60.8 ± 2.9	61.6 ± 2.1	56.0 ± 0.9
Mo(VI)	71.9 ± 2.4	54.5 ± 1.1	51.4 ± 3.4
Mixture[e]	40.2 ± 1.4	NT[f]	NT

[a] Mean values derived from triplicate experiments performed with a single DCAA preparation.
[b] Metals were added to DCAA at 10 μM.
[c] Metals were complexed with pyoverdin in 1:1 molar ratio and added to DCAA at 10 μM.
[d] Metals were complexed with pyochelin in 1:2 molar ratio and added to DCAA at 10 μM.
[e] All listed metals were added to DCAA at 10 μM each.
[f] NT, not tested.

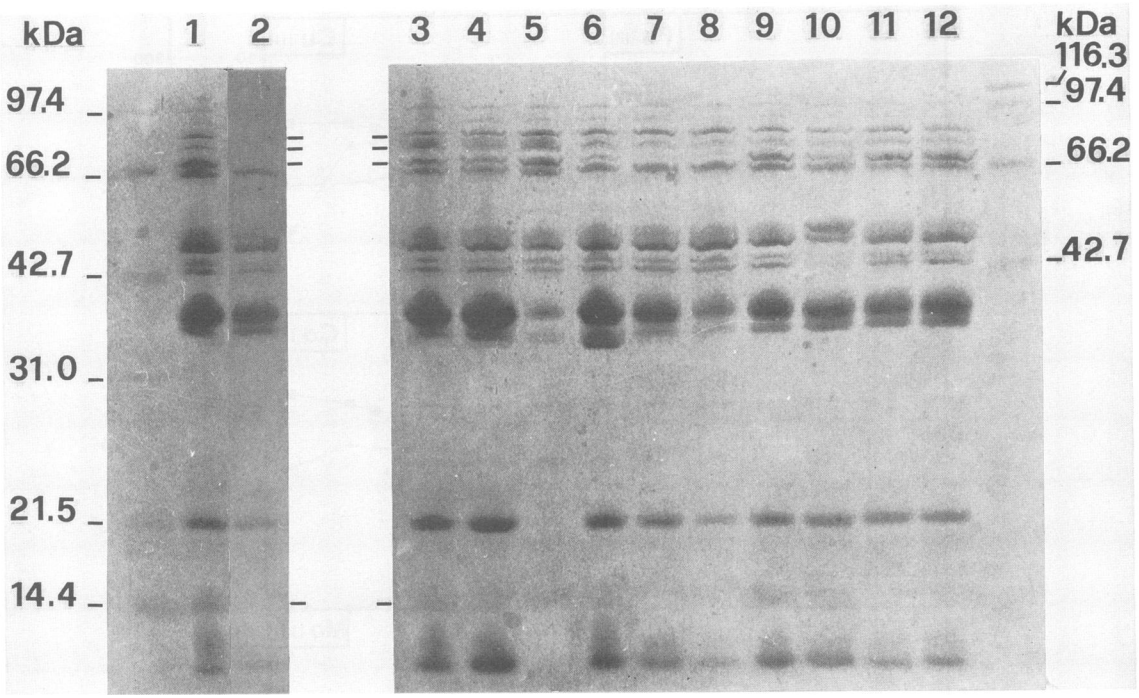

FIG. 2. SDS-PAGE of OMPs from *P. aeruginosa* PAO1. Lanes: 1, OMPs in metal-poor medium (DCAA); 2, OMPs in DCAA supplemented with 100 μM Fe(III); 3 and 4, OMPs in DCAA supplemented with 10 and 100 μM Cu(II), respectively; 5 and 6, OMPs in DCAA supplemented with 10 and 100 μM Ni(II), respectively; 7 and 8, OMPs in DCAA supplemented with 10 and 100 μM Co(II), respectively; 9 and 10, OMPs in DCAA supplemented with 10 and 100 μM Zn(II), respectively; 11 and 12, OMPs in DCAA supplemented with 10 and 100 μM Mo(VI), respectively. Molecular mass standards including *Escherichia coli* β-galactosidase (116.3 kDa), rabbit muscle phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg-white ovalbumin (42.7 kDa), bovine carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg-white lysozyme (14.4 kDa) are shown on sides of the gel. The positions of relevant proteins is indicated between lanes 2 and 3. About 30 μg of proteins was loaded in each lane.

plexes with Fe(III) and Mo(VI) and the pyochelin complexes with Ni(II), Mo(VI), Zn(II), and, to a smaller extent, with Co(II) and Fe(III) showed statistically significant effects. In general, the pyochelin complexes were more effective than their pyoverdin counterparts. This is of particular interest for the pyochelin complexes with Co(II) (t = 2.923; P = 0.043) and Mo(VI) (t = 1.501; P = 0.207), because of the modulatory activity of these metals on pyochelin biosynthesis. Remarkably, the highest growth stimulatory effect was observed by addition of a mixture of all metals at 10 μM each, suggesting that *P. aeruginosa* growth in DCAA is limited by the reduced availability of a number of transition metals. Finally, no significant variation in the generation time was observed upon addition of Mn(II) either as a metal salt or as a siderophore complex (data not shown).

Effect of different transition metals on the OMP profile of *P. aeruginosa* PAO1. A change in the *P. aeruginosa* OMP composition has been shown to occur during iron-limited growth (29). Therefore, we analyzed the OMP profiles in cells grown in DCAA supplemented with different transition metals (Fig. 2). Lanes 1 and 2 show the OMP profile obtained from PAO1 cultures in metal-poor and iron-supplemented media, respectively. Under metal-restricted conditions, at least three high-molecular-mass OMPs of 68, 75, and 80 kDa and a low-molecular-mass OMP of 14 kDa are present whose synthesis is repressed in the iron-supplemented sample. These OMPs probably correspond to the ferripyoverdin (80-kDa) and ferripyochelin (14- and 75-kDa) uptake proteins (18, 28, 34). Lanes 3 to 12 show the OMP profiles obtained when transition metals other than iron were added to the growth medium at 10 and 100 μM. The pattern displayed changes in the relative intensity of individual high-molecular-mass OMPs in the presence of the added metals; the most relevant alteration occurred in the 68-kDa OMP, which was barely detectable when cultures were supplemented with 10 and 100 μM Co(II) and 100 μM Zn(II). Also, the expression of the 75-kDa OMP seemed reduced in cultures containing Co(II), Zn(II), and Mo(VI) at 10 and 100 μM. Moreover, some differences were observed at the level of the faint band at 14 kDa which could not be detected when PAO1 was grown in DCAA containing 10 and 100 μM Co(II) and Mo(VI) or 100 μM Ni(II) and Cu(II). The expression of an approximately 48-kDa OMP was also noted with the addition of 100 μM Zn(II), as reported by other workers (1). Thus, repression of pyochelin biosynthesis by Fe(III), Co(II), and Mo(VI) appears to be related to the reduced expression of well-known iron-regulated OMPs, namely, the 75- and 14-kDa proteins involved in ferripyochelin uptake. In contrast, expression of the 80-kDa OMP involved in ferripyoverdin uptake (28) is not affected by metals other than Fe(III). Lastly, changes in the OMPs at approximately 21 and 35 kDa were produced by the addition of Ni(II) at 10 and 100 μM, respectively.

Spectrophotometric titration of pyochelin with transition metals. The results just presented demand the transition metals tested to form complexes of different affinities with pyochelin and led us to perform direct metal binding experiments. Measurements were carried out at pH 2.5, where the

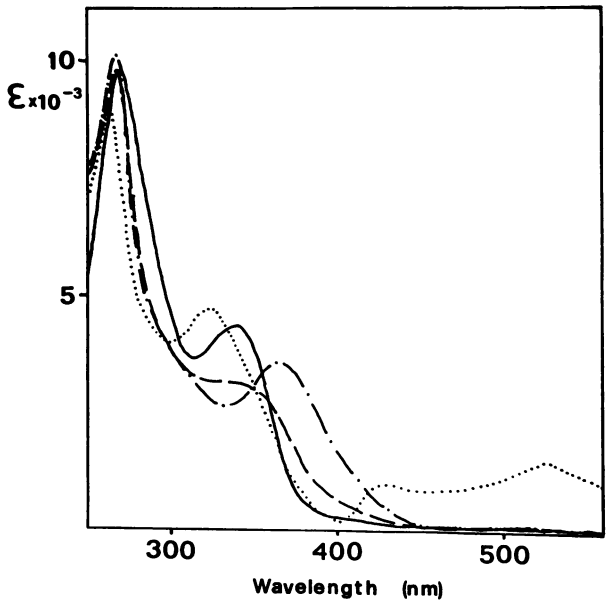

FIG. 3. Absorption spectra of pyochelin upon addition of metals. The solvent was methanol at pH 2.5, and the temperature was 20°C. Symbols: ——, pyochelin; ·····, pyochelin plus $FeCl_3$ [Fe(III)/pyochelin = 1]; – – – –, pyochelin plus $CuSO_4$ [Cu(II)/pyochelin = 1]; — · — · —, pyochelin plus $CuSO_4$ [Cu(II)/Pyochelin = 10].

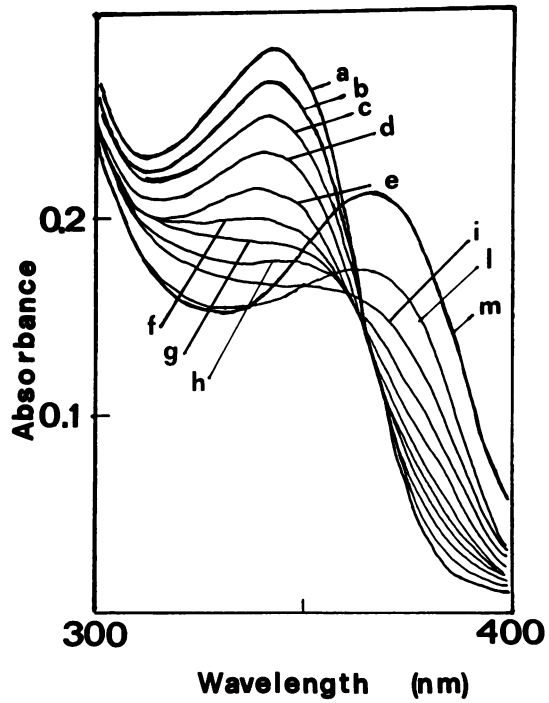

FIG. 4. Titration spectra of pyochelin with Cu(II). A total of 120 nmol of pyochelin in 1 ml of methanol at pH 2.5 was supplemented with 0 (a), 10 (b), 20 (c), 30 (d), 40 (e), 50 (f), 60 (g), 70 (h), 100 (i), 200 (l), and 700 (m) nmol of $CuSO_4$.

solubility of the metals under study is sufficiently high. Moreover, at this pH, iron binding to pyochelin determines significant changes in the optical absorption spectrum of the molecule (10). Thus, metal-free pyochelin at pH 2.5 shows peaks at 208, 270, and 340 nm, whereas the Fe(III)-bound form [one Fe(III) for every two pyochelin molecules] displays maxima at 214, 230, 262, 325, 425, and 520 nm (Fig. 3).

To verify the possible complex formation between pyochelin and metals other than iron, the UV-visible spectra of pyochelin were examined after addition of Mo(VI), Ni(II), Cu(II), Co(II), Zn(II), and Mn(II). The spectral changes observed at different metal/pyochelin ratios suggest the formation of complexes with different stoichiometry, namely one or two metal ions per pyochelin molecule, characterized by distinct spectral properties in the 320- to 400-nm region (Fig. 3). This hypothesis was confirmed in titration experiments. One of these titrations carried out with Cu(II) is shown in Fig. 4. A clear spectral transition occurs when Cu(II) is in excess over pyochelin; under this condition, the complex exhibits an absorption maximum at 366 nm. The other metals studied displayed the same behavior, with the exception of Fe(III), whose relatively high affinity leads to the stabilization of the one metal/two pyochelin (molecule ratio) complex only. The binding affinities for pyochelin decrease in the order Fe(III) $\gg$ Mo(VI) = Cu(II) > Ni(II) > Co(II) > Zn(II) $\gg$ Mn(II), as indicated by the titration experiments plotted in terms of the fractional saturation of pyochelin with the metal (Fig. 5).

DISCUSSION

The striking differences in structure, physicochemical properties, and affinity for ferric iron of pyoverdin and pyochelin, the two siderophores produced by *P. aeruginosa*, suggested to us that one of the two chelators might be involved in the delivery of transition metals other than iron. The suitable candidate was pyochelin, an unusual siderophore because of its remarkably low binding constant for ferric iron and the incomplete iron repressibility of the 14-kDa ferripyochelin-binding protein (27). Furthermore, pyochelin in principle forms an efficient generalized metal ion binding center because of the preponderance of oxygen atoms in the proposed iron ligand groups (hydroxylates and carboxylates); indeed, the ready formation of stable pyochelin-Zn(II) complexes has been reported (13).

Among the various transition metals tested, which are all essential enzymatic cofactors in *P. aeruginosa* (5, 15, 38), Zn(II) and Mn(II) have scarce functional effects. In contrast, Co(II), Mo(VI), Cu(II), and Ni(II) coordinately repress the synthesis of pyochelin and of its 14- and 75-kDa OMPs involved in ferripyochelin uptake but do not significantly affect pyoverdin production or expression of the 80-kDa putative receptor. However, the metal ion concentrations at which these functional effects manifest themselves differ markedly; thus, Co(II) and Mo(VI) are effective at micromolar concentrations, whereas Cu(II) and Ni(II) are required at concentrations which are very far from the physiological ones (approximately 100 μM). In agreement with their modulatory effect on siderophore biosynthesis, these metals [in particular, Co(II) and Mo(VI)] stimulate bacterial growth more efficiently when added to the culture medium in the form of pyochelin complexes than in the form of the respective metal salts or pyoverdin complexes. Both effects parallel the well-known functional roles of Fe(III). Of course, for a given siderophore-metal complex to be functionally relevant, it has to promote growth significantly also in comparison with the siderophore alone. If this comparison is taken into account, only the pyoverdin complexes with Fe(III) and Mo(VI) and the pyochelin complexes with Fe(III), Ni(II),

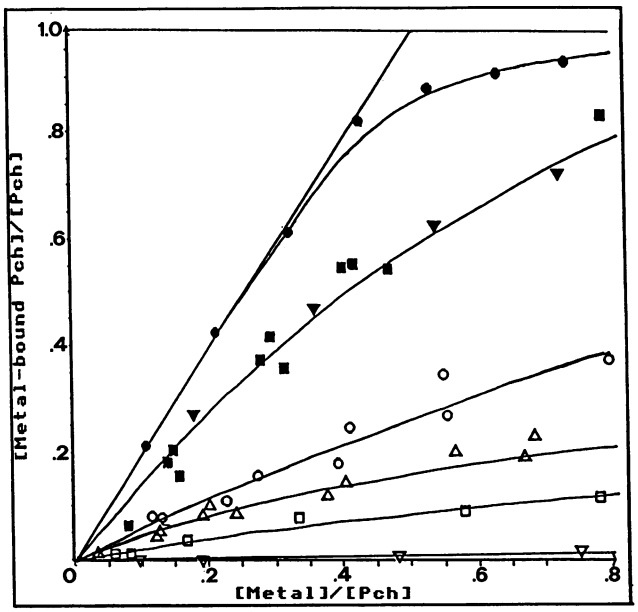

FIG. 5. Fractional saturation of pyochelin with different metal ions. The titrations were performed by adding the metal to pyochelin in methanol at pH 2.5 and 20°C. The fractional saturation was calculated for Fe(III) at 520 nm and for all the other metal ions at 340 nm, where the contributions of the two types of pyochelin-metal complexes are similar. Symbols: ●, Fe(III); ▼, Mo(VI); ■, Cu(II); ○, Ni(II); △, Co(II); □, Zn(II); ▽, Mn(II). Pch, pyochelin.

Co(II), Zn(II), and Mo(VI) should be functional in siderophore-mediated metal transport. The behavior of Mo(VI) is remarkable insofar as its growth-promoting activity depends critically on the mode of administration to the bacterium. Thus, the Mo(VI) complex with pyochelin reduces the mean generation time of *P. aeruginosa* in DCAA to values characteristic of the ferripyochelin complex, while the metal salt [as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$] is very inefficient. This difference may be related to the solution chemistry of molybdenum. The Mo(VI) cation is characterized by an extremely high charge density and reacts with water to form several species, namely MoO_2^{2+} and MoO_4^{2-}, which are in pH-dependent equilibrium. The predominant form in neutral aqueous solutions is MoO_4^{2-} (19), a species unlikely to diffuse easily across the bacterial membrane.

The contention that the functional effects can be ascribed to pyochelin-mediated transport of the various metals is supported by the fair correlation between the concentrations at which such effects are apparent and the metal affinity for pyochelin. The correlation is quite good if one considers that the binding measurements have been carried out at pH 2.5 in methanol (for the reason detailed in Materials and Methods). The metal-pyochelin affinities relative to the divalent ions (Fig. 5) follow the Irving-Williams series and the stability series of the salicylaldehyde-metal (25) or the substituted diketone-metal complexes (36). The higher affinity of Fe(III) with respect to the divalent metal ions is in line with the equilibrium constants of the metal-ligand reactions when the ligand is EDTA, catechol, or salicylic acid (23, 24). The formation of two distinct types of complex (with 1:1 or 1:2 stoichiometry) can be predicted as well on the basis of literature data on the reactivity of phenolates (derivatives of salicylic acid, salicylaldehyde, and catechol, etc.) (24). Moreover, copper and molybdenum have previously been shown to possess appreciable affinities for hydroxamate and catechol ligands (19). The behavior of Mo(VI) can be explained again in terms of the prevalent species in solution. The observed affinity for pyochelin indicates that, under the conditions used, MoO_2^{2+} dominates the experimental situation because this cation forms stable metal-cathecol complexes of stoichiometry 1:2 or 1:1 (21, 31) just like the positive divalent metals do (24). In contrast, MoO_4^{2-} has a surface which is not occupied by oxygen atoms and resembles that of Fe(III); thus, like this cation, MoO_4^{2-} has a high localized charge density and binds anions very strongly (19).

The observed effects on the expression of the OMPs involved in ferripyochelin uptake deserve a comment. The coordinate repression of the synthesis of the 14- and 75-kDa proteins in Co(II)- and Mo(VI)-proficient cells suggests that both the high- and low-affinity systems might be involved in pyochelin-mediated uptake of these transition metals. In this respect, the acquisition of the putative Co(II) and Mo(VI) complexes of pyochelin differs from that of Fe(III), since, for iron, the two uptake systems can be differentiated on the basis of their affinity, specificity, and response to metal starvation (18).

In conclusion, our results show that the functional effects of Fe(III) can be mimicked by other transition metals endowed with a high affinity for pyochelin. Thus, Co(II) and Mo(VI) at micromolar concentrations reduce both pyochelin synthesis and expression of ferripyochelin uptake proteins and promote bacterial growth in a metal-restricted environment when administered as pyochelin complexes. Conversely, Mn(II), which has a much lower affinity for pyochelin, has practically no effect. These considerations lead to the proposal that binding of Co(II) and Mo(VI) to pyochelin may well play a role in their delivery to *P. aeruginosa* cells to counter metal limitations imposed by the growth environment.

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